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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Deregulated receptor-tyrosine kinase (RTK) signalling is a hallmark of many breast cancers and therefore RTKs and their associated signal transducers have emerged as targets for novel therapeutic strategies as well as markers for patient prognosis. Docking proteins of the Gab/Dos family amplify RTK signals and couple these to the Ras/MAPK and PI-3K pathways, two major signaling pathways with well established roles in tumorigenesis. Expression of the Gab2 isoform is often elevated in primary breast cancer specimens and is positively regulated by estrogen. This suggests that Gab2 plays a role in the crosstalk between steroid hormone and growth factor receptors pathways, which is implicated in the decreased estrogen sensitivity and anti-estrogen resistance of breast cancer cells. We have therefore initiated the present project to analyse the importance of Gab2 over-expression for the development and progression of breast cancer. Within the last year, we have established Gab2 overexpressing HC11 cells, a widely used model system for the analysis of signaling pathways in normal mammary epithelium. We report here the phenotypic analysis of Gab2 overexpressing HC11 cells in terms of growth factor- and serum-induced proliferation. Furthermore, we have established stable Gab2 expressing pools of the estrogen-dependent breast cancer line ZR-75-1.

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The role of the Gab2 docking protein in human breast cancer

Introduction

Deregulated receptor-tyrosine kinase (RTK) signalling is a hallmark of many breast cancers and therefore RTKs and their associated signal transducers have emerged as targets for novel therapeutic strategies as well as markers for patient prognosis (*1*). Among the important RTK signal transducers are the docking proteins of the Gab/Dos family. Gab proteins amplify and integrate RTK derived signals and couple these to downstream situated cytoplasmic signalling pathways like the Ras/Mitogen-activated protein kinase or Phosphatidyl-inositol-3 kinase/protein kinase B (PKB) pathways, two major signalling pathways with well established roles in malignant transformation (*2-5*). By modulating the extent of the activation of these pathways, Gab proteins play a crucial role in the fine-tuning of these key signals that ultimately decide over cell growth, proliferation, survival and differentiation (*6-8*). Furthermore, the expression and phosphorylation of Gab proteins appears to be a limiting factor in the extent of RTK-mediated activation of the aforementioned pathways (*9, 10*). Genetic studies in mice have also demonstrated unique and redundant functions for the individual Gab isoforms during development (*11-13*).

Gab proteins have been also directly implicated in oncogenic signalling. Gab2, in particular, has been shown to play a pivotal role in the transformation of hematopoietic cells and fibroblasts by the Bcr-Abl (*14*) and v-SEA oncoproteins (*15, 16*), respectively. Interestingly, the *GAB2* gene is located on human chromosome11q13.3–14.2, (*17*), a chromosomal region found to be amplified in 10–15% of breast cancers (*18*) and, indeed, elevated Gab2 expression levels have been found in primary breast cancer specimens (*19*). Furthermore, Daly *et al.* (2002) demonstrated that Gab2 expression is positively regulated by estrogen. Positive regulation by estrogen suggest that Gab2 may play a role in the crosstalk between steroid hormone and growth factor receptors pathways (*20, 21*). Overexpression of Gab2 may amplify RTK signalling at two points: Firstly, increased Gab2 levels may improve proximal RTK signalling itself. Secondly, amplification of RTK induced MAPK and PI-3K/PKB signalling may lead to enhanced

steroid induced transcription through phosphorylation of the estrogen receptor (ER) α. These observations suggest that increased RTK signaling might lead to decreased estrogen sensitivity of breast cancer cells and ultimately anti-estrogen resistance (for review see (*19, 21*)). Lastly, Gab2 is subject to a feedback phosphorylation by activated PKB at S159 and the $Gab2^{S159A}$ mutant, which is consequently uncoupled from this negative feedback loop, displays transforming properties in fibroblasts (*9*). Taken together, these studies establish Gab2 as a potential oncogene and we have therefore initiated the present project to analyse the importance of Gab2 (over)expression for the development and progression of breast cancer. Given the overexpression of Gab2 in many breast cancers, we are particularly interested as to whether its ectopic expression alters RTK signalling in Gab2 negative or low expressing mammary epithelial cell lines. Furthermore, we want to determine whether Gab2 (over)expression alters estrogen responsiveness and dependence in ER-positive breast cancer cell lines derived from mammary epithelium and tumours. Ultimately, the current study might help to establish Gab2 as a novel prognostic marker and/or identify it as a novel therapeutic target for breast cancer with deregulated RTK-signalling.

Body

Task 1: Generation of stable HC11 pools over-expressing Gab2 (month 1 to 2)

Aim: • Infection of HC11 cells with Gab2 encoding retrovirus and selection of stably overexpressing pools

The immortalised mouse cell line HC11 is a widely used model system for the analysis of hormone- and growth factor (GF) action in non-transformed mammary epithelium (*22*) and displays only weak Gab2 expression (our unpublished results). Therefore, we asked as to whether Gab2 overexpression would influence growth factor signalling and biological endpoints like proliferation. To this end, we generated stable HC11 pools that have been infected with an ecotropic retrovirus encoding Gab2 (pJZEN-Gab2-neo; (*9*)). As a control, HC11 pools infected with the empty retroviral construct were also generated (pJZEN-neo). Following selection with neomycin, several independent pools were assessed for Gab2 expression by Western blotting (**Figure 1**). Two independent pools with comparable Gab2 expression were chosen for further phenotypic analysis (see Task 3).

Task 2: Generation of stable ZR-75-1 pools over-expressing Gab2 (month 1 to 6)

Aim: • Transfection of ZR-75-1 cells with EcoR plasmid

- Selection of EcoR-expressing clonal cell line
- Confirmation of phenotype of clonal cell line
- Infection of line with Gab2 retrovirus
- Selection of stably Gab2 overexpressing pools

Given the well documented crosstalk between RTK and ER signalling at various levels in both pathways (*20, 21*), we also sought to analyse the effects of Gab2 expression on

GF- and estrogen-induced signalling in the ER-positive but Gab2-negative breast cancer cell line ZR-75-1 (*19, 23, 24*). As ZR-75-1 cells were reported to display poor transfection efficiency, we tried the aforementioned retroviral expression vectors to establish stable Gab2 expressing ZR-75-1 pools. As ZR-75-1 cells are of human origin and therefore lack the membrane protein used by ecotropic rodent retroviruses (ecoR; (*25*)) for host cell infection, we first established a stable ZR-75-1 line expressing the ecoR protein by conventional transfection. Several independent clones were obtained and screened for their potential to be infected by ecotropic retroviruses encoding Enhanced Green Fluorescent Protein (EGFP). One clone, which is designated as ZR-75-1 B1, displayed the highest but still low (15 % or less cells GFP positive cells) infection rate and was chosen for further experiments. As extensive studies in our laboratory have shown, ZR-75-1 B1 proliferates in response to E2 and shows similar sensitivity towards anti-estrogens as the parental ZR-75-1 line (**Figure 2**).

We then tried to establish Gab2 expressing ZR-75-1 B1 cells by retroviral infection. Although ZR-75-1 B1 cells could be infected with ecotropic retroviruses to a low extent, we failed to establish stable neomycin resistant pools in three independent experiments. Therefore, we tried to establish Gab2 expressing ZR-75-1 cells by conventional plasmid transfection. To this end, we transfected ZR-75-1 B1 cells with either pCDNA3.1 (Invitrogen) or pCDNA3.1/Gab2. Following neomycin selection, we obtained two independent pools stably expressing Gab2 (**Figure 3**), which are currently being analysed as proposed in tasks 3 – 5.

Task 3: Characterization of the serum and growth factor sensitivity of the HC11 and ZR75-1 pools (months 7 to 10)

Aim: • Analysis of the serum sensitivity of the HC11 pools under anchorage dependent and independent conditions

- Characterization of the growth factor sensitivity of the HC11 pools
- Analysis of the growth factor sensitivity of the ZR-75-1 pools

i.) HC11

We analysed the serum sensitivity of Gab2 expressing HC11 pools under anchorage-dependent conditions. Despite its prominent overexpression (**Figure 1**), we observed only a marginal effect of Gab2 on proliferation induced by 2 % serum, while effects were not observed at other serum concentrations (**Figure 4A**). Next, we analysed the responsiveness of Gab2 overexpressing HC11 pools towards various epidermal growth factor (EGF), β-Heregulin (HRG) and insulin (**Figure 4B**). However, we could not observe a prominent influence of Gab2 overexpression on the growth factor induced proliferation of HC11 pools, although a small enhancement on EGF-induced proliferation was observed in some experiments. Significant effects of Gab2 overexpression were also not observed when different concentrations of EGF and HRG were used (**Figure 5**). Similar results were also reproduced using a second independent set of HC11 pools (data not shown). Given the marginal effect of Gab2 overexpression for serum-induced proliferation of HC11 pools, we refrained from analysing their serum sensitivity under anchorage-independent conditions as originally planned. It might be possible that the endogenous Gab2 expression levels are already sufficient to mediate a maximum response to serum and growth factors that cannot be further increased by Gab2 overexpression. During the course of these experiments, we have also noticed some morphological heterogeneity and changes in Gab2 expression levels between the different pools. We attribute these effects to a putative phenotypic drift induced by Gab2 overexpression and/or growth conditions during the selection phase over several weeks. Based on these experiences, we therefore conclude that HC11 cells are not a suitable model system for the analysis of Gab2 overexpression on the growth characteristics of non-transformed mammary epithelial cells. Consequently, we have searched the literature for a different, Gab2-negative model system and have chosen the well-documented, non-transformed human mammary epithelial cell line MCF-10a cells for initial pilot experiments. MCF-10A cells originate from breast tissue of a 36-year-old patient with fibrocystic changes and have undergone spontaneous immortalisation in culture. These cells display numerous hallmarks of normal breast epithelium, including lack of tumorigenicity in nude mice, lack of anchorage-independent growth, and the requirement for growth factors and

hormones for proliferation and survival (*26, 27*). Furthermore, after 15 days in three-dimensional culture in matrigel, MCF-10A cells form acinar structures that retain important characteristics found in glandular epithelium *in vivo*, like apico-basal polarization, suppression of proliferation and acinar cavitation through apoptosis of excess cells located within the inner cell mass (*27-30*). By using this system Debnath *et al.* (2002) were able to show that "proliferative" oncogenes like Cyclin D1 override the proliferative suppression during acinus formation but are unable to block apoptosis and acinar cavitation, whereas active ErbB2 drives both proliferative and anti-apoptotic programs and therefore disrupts acinus development completely (*28*). Thus, this system allows the characterization of signaling molecules or (potential) oncogenes in terms of their impact on cell cycle progression, apoptosis, cellular polarity and cell-cell as well as cell-matrix adhesion. Furthermore, this system is suitable for the molecular dissection of cellular processes observed during the formation of atypical hyperplasias and ductal carcinoma *in situ* (DCIS; (*28*)) and consequently provides more information than simple focus-forming or soft-agar assays. We were therefore highly interested to assess the influence of Gab2 (and mutants thereof) in this system, which appears to us in many aspects as superior over the HC11 system.

In initial experiments, we analysed the expression levels of Gab1 and Gab2 in MCF-10a cells. In line with the finding by Daly *et al.* (2002), the ER-negative MCF-10a cells (*27*) express the ubiquitously expressed Gab1 isoform but not Gab2 (data not shown) and are therefore a suitable, if not better replacement for the HC11 system. Based on our experience with the phenotypic drift of the stable HC11 pools, we decided to analyze freshly infected, Gab2 expressing MCF-10a cells in order to follow the effect of Gab2 expression from an early stage. To this end, we subcloned the human Gab2 cDNA into the bi-cistronic retroviral expression vector pMIG (*31*) allowing the co-expression of C-terminal hemagglutinine-(HA)-tagged Gab2 and EGFP from the same transcript (**Figure 6A**). This strategy allows us to identify Gab2 expressing cells by their green autofluorescence and to purify them to homogeneity by flow cytometry. In initial pilot experiments, we infected MCF-10a/ecoR cells, a transfected MCF-10a subclone expressing the receptor for murine retroviruses (a kind gift by Dr. D. Lynch, Boston), with ecotropic pMIG or pMIG/Gab2 virions. As shown in **Figure 6B**, MCF-10a/ecoR cells

were successfully infected and EGFP-expressing cells can be easily identified by flow cytometry. Currently, we are using mixed and sorted populations for the analysis of their growth factor sensitivity and their potential to establish epithelial acini. We will therefore this as an alternative to HC11 cells for examining the effects of Gab2 overexpression on normal mammary epithelial cells

ii.) ZR-75-1

Due to the initial problems with establishing stable Gab2 expressing ZR-75-1 B1 pools (see task 2), the proposed experiments for task 3 have not started yet.

Task 4: Characterization of the estrogen/anti-estrogen sensitivity of the ZR-75-1 pools (months 11-14)

Aim: • Analysis of estrogen sensitivity under anchorage dependent and independent conditions

- Analysis of anti-estrogen sensitivity

Due to the aforementioned initial problems with the ZR-75-1B1 cells (see task 2), the proposed experiments for Gab2 expressing ZR-75-1 pools have just begun.

Task 5: Analysis of signaling pathways in the HC11 and ZR-75-1 pools (months 15 – 17)

Aim: • Characterization of ERK and PKB activation

- Determination of Ras and PI-3K activation
- Analysis of ER activation via transient transfection

As the Gab2 (over)expressing HC11 and ZR-75-1 pools are now established, the analysis of signaling pathways in these cells will start shortly. However, as outlined

above, we think that the HC11 system is not very suitable for this part of the project and we will also include the MCF-10a system for the analysis of the impact of Gab2 on RTK-signaling in normal mammary epithelial cells.

Key Research Accomplishments

- Establishment of Gab2 over-expressing HC11 cells and ZR-75-1 cells

- Demonstration that Gab2 overexpression in HC11 cells only has modest effects on serum- and EGF-induced proliferation.

- Positive evaluation of MCF-10a cells as a suitable substitute for HC11 cells for the analysis of Gab2 expression in normal mammary epithelial cells.

- Generation of a bi-cistronic retroviral vector encoding Gab2.

- Establishment of a protocol for the infection of human MCF-10a cells with ecotropic rodent retroviruses. The protocols to infect MCF-10a cells with pMIG retroviruses are also of general interest to the Cancer Research Program of the Garvan Institute of Medical Research and will be useful for other Garvan researchers involved in the DOD BCRP.

Reportable Outcomes

Reagents:

The following constructs/reagents generated during the course of the project will be useful to scientists performing basic research on Gab2:

- A bi-cistronic retroviral vector encoding Gab2

- Stable Gab2 expressing ZR-75-1 and HC11 pools, which can be used for biochemical assays addressing the interaction of this docking protein with known and unknown interaction partners.

Conclusions

As outlined in the research proposal, we planned the establishment and phenotypic characterisation of Gab2 overexpressing HC11 and ZR-75-1 pools for the first year. We have successfully established these cell lines and have begun their characterisation in terms of growth factor, serum and hormone sensitivity. Whereas the characterisation of the ZR-75-1 pools is still in process, we have addressed these questions in the HC11 system. However, we found that Gab2 overexpression has only a marginal effect on the proliferative behaviour of these cells towards growth factors and serum. Among other possibilities, we reason that the endogenous Gab2 expression levels are sufficient to mediate a maximum response to serum and growth factors that cannot be further increased by Gab2 overexpression. Therefore, we have started to analyse the effects of Gab2 expression in the Gab2-negative non-transformed human mammary epithelial cell line MCF-10a.

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Appendices:

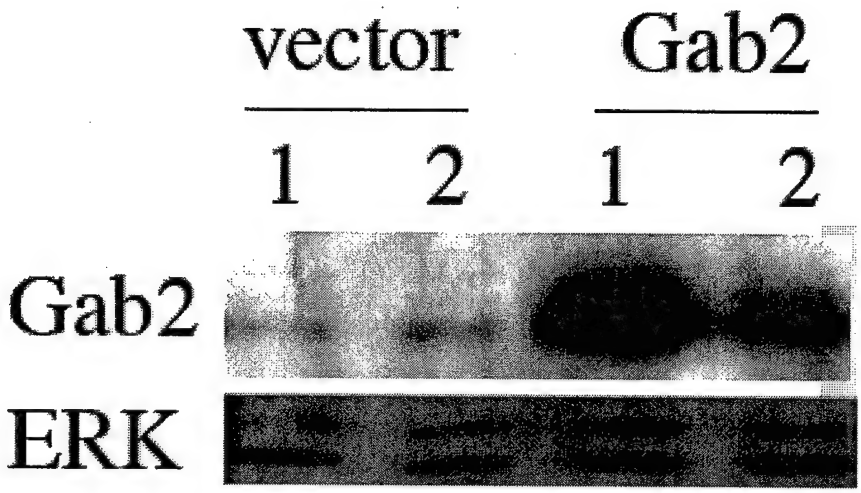

Figure 1. Stable Gab2 expression in HC11 cells. HC11 cells were infected with either pJZEN-neo (vector) or pJZEN-Gab2-neo (Gab2) retroviruses and infected cells were positively selected with neomycin. Lysates from two independent neomycin-resistant pools were analysed for Gab2 expression by Western blotting using an anti-Gab2 antibody (M-19: Santa Cruz). Detection of extracellular signal regulated kinases 1 and 2 (ERK) served as a loading control.

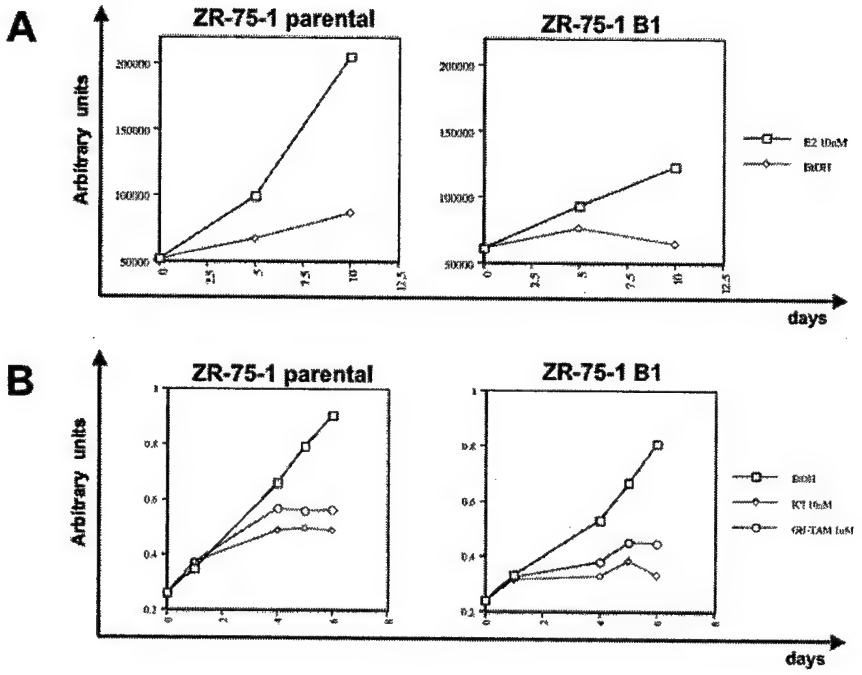

Figure 2. Phenotypic comparison of parental ZR-75-1 cells with ZR-75-1B1 cells expressing the ecotropic receptor for infection with murine retroviruses (A) Estrogen-responsiveness. Parental ZR-75-1 and ZR-75-1 B1 cells were grown in the presence of estrogen (E2) or ethanol (vehicle control). Cell growth was monitored using a MTT assay **(B) Sensitivity towards anti-estrogens.** Parental ZR-75-1 and ZR-75-1 B1 cells were grown in the presence of estrogen (E2) and in combination with either ethanol (vehicle control) or the anti-estrogens ICI and 4-hydroxy-tamoxifen (OH-TAM). Proliferation was measured using a MTT assay.

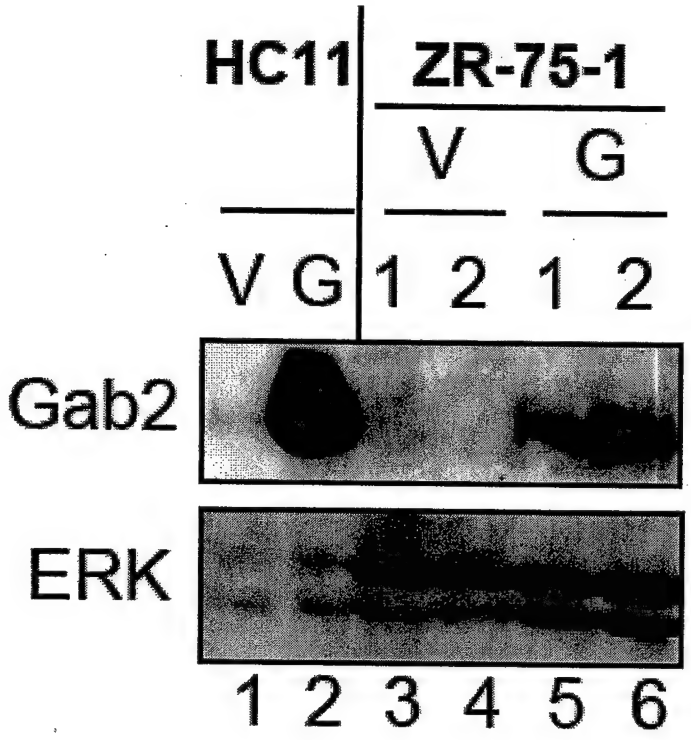

Figure 3. Stable Gab2 expression in ZR-75-1 B1 cells. ZR-75-1 B1 cells were transfected with either empty vector pCDNA3.1 (V) or pCDNA3.1/Gab2 (G) and selected with neomycin. Following neomycin selection, cells from two independent pools were lysed and subjected to a Western blot analysis using an anti-Gab2 antibody (M-19, Santa Cruz Biotechnologies). For a better evaluation of the Gab2 expression levels in ZR-75-1 B1 pools, lysates from pJZEN-neo infected HC11 cells (V, lane 1) with low levels of endogenous Gab2 and from pJZEN-Gab2-neo infected cells (G, lane 2) expressing high levels of ectopic Gab2 were included in this analysis. Detection of ERK1,2 served as a loading control.

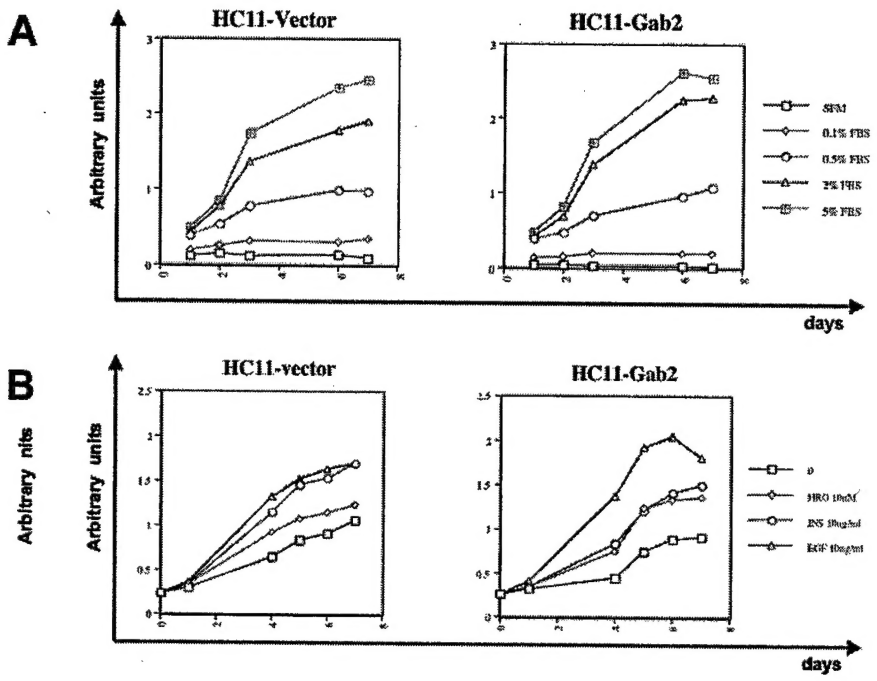

Figure 4. Effect of Gab2 overexpression on serum- and growth factor induced proliferation of HC11 cells. (A) Serum-induced proliferation. HC11 cells were serum-starved and then exposed to the indicated serum (FBS) concentrations. Proliferation was measured using a MTT assay. **(B) Growth factor-induced proliferation.** HC11 cells were serum-starved and then exposed to the indicated concentrations of growth factors in the presence of 0.25 % fetal bovine serum (FBS). Proliferation was measured using a MTT assay.

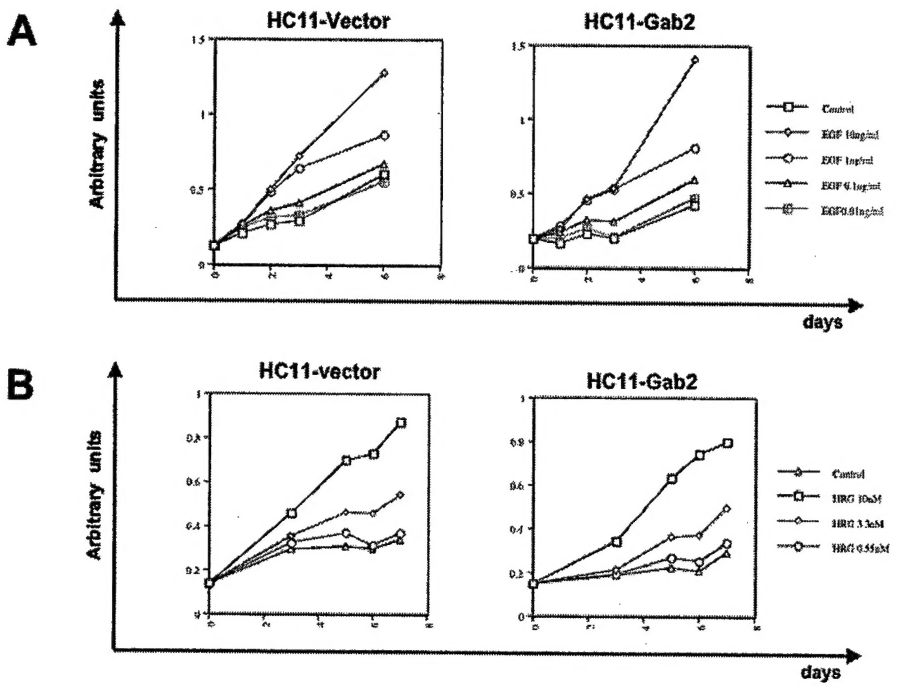

Figure 5. Effect of Gab2 overexpression on HC11 proliferation induced by various amounts of EGF and HRG. HC11 cells were serum-starved and then exposed to the indicated GF concentrations (in the presence of 0.25 % FBS). Proliferation was measured using a MTT assay. **(A) EGF -induced proliferation. (B) HRG -induced proliferation.**

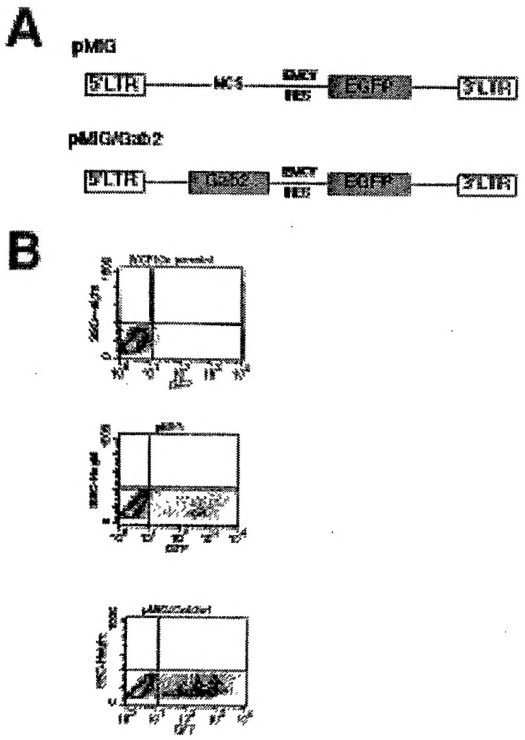

Figure 6 Infection of MCF-10a cells with a bicistronic retrovirus encoding both Gab2 and EGFP. (A) Overview of the retroviral constructs used for ectopic Gab2 expression in MCF-10a cells. The vector backbone pMIG (also known as pMSCV-IRES-EGFP: (*31*)) is derived from the murine stem cell virus (MSCV) and contains a multi-cloning site (MCS) followed by internal ribosomal entry site (IRES) derived from the equine meningoencephalitis virus (EMCV) and an EGFP cassette (green box). The vector pMIG/Gab2 was generated by subcloning the coding sequence for C-terminally HA-tagged human Gab2 (orange box) into the MCS of pMIG. The vector pMIG/Gab2 encodes for a bi-cistronic mRNA encoding both Gab2 and EGFP. Translational re-initiation and EGFP expression is mediated by the IRES sequence. **(B) Flow cytometric analysis of MCF-10a/ecoR cells infected with pMIG virions.** MCF-10a/ecoR cells were incubated with

supernatants from the retroviral packaging phoenix cell line, which was either transfected with pMIG (middle panel) or pMIG/Gab2 (lower panel) vectors as described (27). Following 8 days in culture, the infected MCF-10a/ecoR pools were analysed for EGFP expression by flow cytometry.